Interaction of Diltiazem with Single L-type Calcium Channels in Guinea-pig Ventricular Myocytes

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Abstract. The effects of diltiazem on cardiac L-type calcium channels were studied at the single channel level, using Ba^{2+} ions as the charge carrier. Patch clamp experiments were performed on enzymatically isolated guinea-pig ventricular myocytes. It was shown in cell-attached configuration on multichannel patches that diltiazem, when applied to the bath, can approach the calcium channel under the pipette after diffusion through the membrane phase. The time constant of the onset of the effect was 60 s. The rate of recovery seemed to be of the same order. Diltiazem had most prominent effect on calcium channel open state probability by reducing the frequency of openings, and by increasing the frequency of records without channel opening (nulls). The effect on mean open time was found to be insignificant at 1 kHz resolution. Diltiazem had no effect on the amplitude of unitary currents. These data are consistent with the assumption that diltiazem interacts mainly with the inactivated state (although interaction with the closed states was not ruled out), and does not bind to the open state of the calcium channel.

Key words: Diltiazem — Calcium channel — Patch clamp — Ventricular myocytes — Guinea pig

Introduction

L-type calcium channels of heart muscle cells are important targets for organic calcium antagonists. Three therapeutically important types of these agents have been described: dihydropyridines, diphenylalkylamines, and benzothiazepines (Glossmann et al. 1983; Triggle 1987). According to voltage-clamp studies, these agents differ in the degree of their use-dependence: diphenylalkylamines are strongly use-dependent, dihydropyridines are poorly use-dependent, and benzothiazepines are intermediate in this respect (Lee and Tsien 1983; Uehara and Hume 1985).

On the basis of the modulated receptor hypothesis (Hondenghem and Katzung 1984) this was ascribed to the differences between relative affinities of individual antagonist types to the resting, open and inactivated channels states (Pelzer et

al. 1982; Lee and Tsien 1983; Tung and Morad 1983; Bean 1984; Sanguinetti and Kass 1984; Uehara and Hume 1985; Klöckner and Isenberg 1986). All three groups of Ca antagonists have been shown to interact with the inactivated state of Ca channel; on the other hand, their use-dependence was ascribed to interaction with either the open channel (Klöckner and Isenberg 1986), or with the inactivated channel (Kanaya et al. 1983), or both (Lee and Tsien 1983). Whole-cell data indicated that dihydropyridines, which do not display use-dependent effect, may also interact with open Ca channel (Lee and Tsien 1983; Uehara and Hume 1985). It was noticed, however, that measurements of macroscopic currents cannot give a definite answer to this problem (Tung and Morad 1983; Hondenghem and Katzung 1984) and single channel experiments are required.

So far, the interaction with single Ca channels has been studied for two calcium antagonist types only. Using the method of patch clamp, the inhibitory dihydropyridines nitrendipine and (R)- 202-791 (Hess et al. 1984; Kokubun et al. 1986; Kawashima and Ochi 1988) and the diphenylalkylamine D600 (Pelzer et al. 1984) were found to have many similarities that point to their interaction with inactivated state. On the other hand, the mean open time of calcium channels was found to be decreased by the compound D600 but not by the dihydropyridines. The decrease of the mean open time directly demonstrates interaction of this drug with the open state of calcium channels.

The mechanism of benzothiazepine action has not yet been studied at the level of unitary channels. We aimed to find the effect of the benzothiazepine compound diltiazem and compare it with the data published on other Ca antagonists. Part of this work has already been published in abstract form (Zahradníková et al. 1989).

Materials and Methods

The procedure used for enzymatic isolation of cells was described in detail elsewhere (Zahradník and Zahradníková 1989). Briefly, guinea pigs (200–400 g) were anesthetized, the heart was quickly excised and mounted on a Langendorff column. Retrograde perfusion started with Tyrode solution, then with nominally Ca^{2+}- free Tyrode solution, and finally with the same solution containing 1 mg/ml collagenase, 0.4 mg/ml hyaluronidase (both from ÚSOL, Czechoslovakia), 500 TIU/ml aprotinin (Léčiva, Czechoslovakia), 10 mg/ml albumin (Imuna, Czechoslovakia), and 20 mmol/l taurine (Sigma, USA). All solutions were saturated with 100% O_2 and warmed to 37 °C. Dissociated cells from the left ventricle were filtered through a nylon mesh, and gradually transferred to minimal essential medium (Eagle) (ÚSOL, Czechoslovakia) with 10% fetal calf serum (Institute for Veterinary Studies, Brno, Czechoslovakia). The isolated cells were washed and stored in physiological saline at room temperature during the day of experiment.

Cell suspension was placed into experimental chamber filled with Tyrode solution. Sedimented cells were then slowly superfused with depolarizing solution (in mmol/l): 140 K-methanesulfonate, 10 $MgCl_2$, 10 HEPES, 10 EGTA, pH 7.35. All experiments were performed at room temperature.

The external solution with diltiazem (Sigma, USA, or Lachema, Czechoslovakia) was prepared shortly before the experiments as diltiazem in aqueous solution slowly decomposes (Davis et al. 1986).

Single-channel currents were measured using the technique of patch clamp (Hamill et al. 1981; Sakmann and Neher 1983). Pipettes from borosilicate glass were pulled to 1.5–2 μm o.d., coated with Sylgard 184 (Dow Corning, USA) and heat-polished to have a resistance of $\simeq$ 3 MΩ when filled with the pipette solution (in mmol/l: 110 $BaCl_2$, 10 HEPES, pH 7.4). Patch currents were recorded using a List Electronic EPC-7 device (Darmstadt, FRG), electronically compensated for linear current components, and low pass filtered at a cutoff frequency of 1 kHz. The data acquisition and analysis were carried out on a minicomputer SM 4-20 as described elsewhere (Karhánek et al. 1987; Zahradníková 1987). In short, data were sampled by a 12-bit ADC at 5 kHz sampling rate (1024 data points per sweep). The passive current components were further compensated for by subtracting the average of "nulls" from the records. Events, including multiple ones, were detected using a 50% threshold criterion (the threshold was placed midway between the levels $n-1$ and n) and their amplitude and duration were measured. Amplitudes of single channel currents were determined either as the mean of event amplitudes, or from cumulative amplitude histograms. The number of openings per sweep and open state probability in the sweep were calculated. The number of channels in a patch was estimated using binomial analysis according to Patlak and Horn (1982) and was found to be the same as the observed maximal number of simultaneously open channels. Mean open times were calculated by two procedures: either all multiple openings were discarded, as in Sachs et al. (1982), or events leading to multiple openings were treated as in Kunze et al. (1985). Then, histograms of open times were constructed and fitted by the minimum χ^2 method. Both procedures gave identical results.

Results

The most common way used to study the effects of drugs at the single channel level is the use of one-channel patches. A disadvantage of this approach is in its complexity both for the data acquisition and analysis, especially if a particular channel has a complicated structure so that interpretation of the measured time distributions is dependent on the preferred kinetic model. Of course, this is outweighed by the high information content of the data. However, if detailed information about the closed states distribution is not of prime interest, an alternative approach utilizing multichannel patches can be the matter of choice. In the latter case observation of the time course of drug action is made easier and still some of the advantages of single channel studies are preserved.

This consideration led us to work with multichannel patches as we aimed to identify the principal effects of diltiazem only. The potential of the patches was held at −80 mV and depolarizing pulses (160 ms, +10 mV) were applied in 3.5 s intervals. The rest of the cell membrane was depolarized to 0 mV by the high potassium containing bath solution (Nilius et al. 1985). After measuring a series of control sweeps (usually about 50), the solution in the experimental chamber was replaced with the diltiazem-containing one. A comparatively high concentration of

diltiazem (10 μmol/l) was used to make sure that all its effects will be pronounced.

It should be stressed that in this experimental configuration the observed channels are located under the pipette opening, and the pipette solution that is in contact with the extracellular part of the channel is chemically isolated from the bath (Hamill et al. 1981). Thus, diltiazem, when added to the bath, can exert its effect on the measured Ca channel only after having diffused laterally through and/or across the membrane phase.

The changes brought about by a 10-min presence of diltiazem and by subsequent washout are illustrated in Fig. 1. No change in the amplitude of unitary currents was ever observed (5 experiments). Amplitude of i_{Ba} was 1.1 ± 0.2 pA both in controls and in the presence of diltiazem and corresponded to a single channel

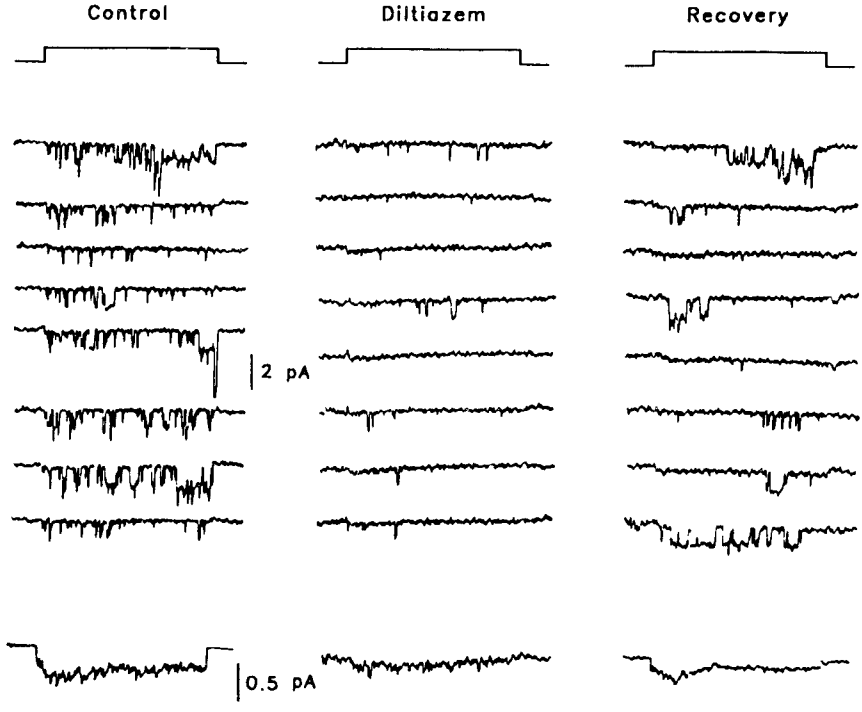

Figure 1. Effect of diltiazem on single L-type calcium channels. Single channel Ba^{2+} currents recorded in response to 160 ms pulses from -80 to $+10$ mV (shown schematically in top row). The left hand panel shows control activity before diltiazem addition (three channels in the patch); the middle panel shows steady state activity in the 9th minute after addition of 10 μmol/l diltiazem to the bath; the right hand panel shows channel activity in the 6th minute after diltiazem washout. Bottom row: Averaged current traces corresponding to the above panels. The traces were obtained by averaging all the records at given experimental conditions (48, 23, and 105, respectively).

conductance $g = 25$ pS (Zahradník and Zahradníková 1989).

On the other hand, the activity of Ca channels in the presence of diltiazem was significantly lower. In principle, the decrease in channel open probability in the sweeps can be a result of changes in the mean open time (τ_0) and/or the mean number of openings per sweep (n_0). The former was not the case as the open time distributions in control and diltiazem were not significantly different (0.61 ± 0.02 vs. 0.57 ± 0.04, mean $\pm$ S.E.M., $n = 3$, $\alpha > 0.1$). This is documented in Fig. 2 for the illustrated experiment, where $\tau_0 = 0.62$ ms for control, diltiazem and recovery. This result means that the open state of Ca channels was not influenced to a measurable extent by diltiazem.

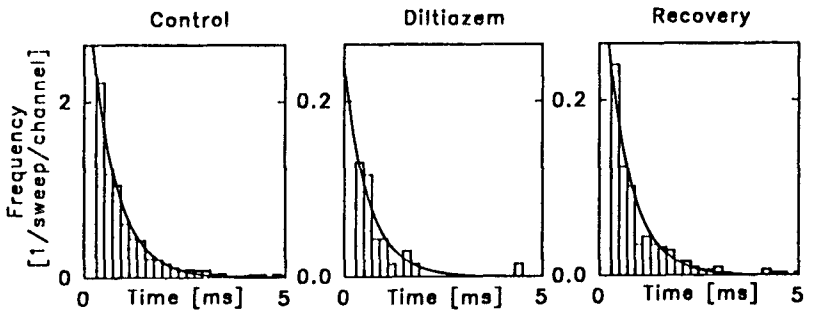

Figure 2. Effect of diltiazem on the distribution of calcium channel open times. Histograms of open times for a channel in control conditions (left), in the presence of 10 μmol/l diltiazem (middle) and after diltiazem removal (right). The histograms are calibrated in units of number of openings per sweep per channel. The solid lines are mono-exponential fits to the distributions. All three histograms could be fitted with the same mean open time $\tau_0 = 0.62$ ms. The areas under the theoretical curves were (in events per sweep per channel): Control, $W_{oc} = 5.9$; diltiazem, $W_{od} = 0.38$, recovery, $W_{or} = 0.99$.

The decreased frequency of channel opening was manifested, first, by occurrence of sweeps without channel activity. The so-called nulls, not observed with multichannel patches under our control conditions, became prominent in the steady state of diltiazem action (22% nulls). Second, the number of events was lowered from 9.8 ± 0.9 (mean $\pm$ S.E.M.) openings per sweep per channel in controls to 5.5 ± 1.5 in the first three minutes after the onset of diltiazem action, and finally down to 0.4 ± 1.2 in the steady state. Multiple openings, which were always frequent before diltiazem addition (21% of control sweeps), completely vanished.

The changes in channel activity after addition of diltiazem are illustrated in Fig. 3 by plotting the open probability in the sweep $\bar{p}$ (Hess et al. 1984) for each sweep during the experiment. To visualize the time course of open probability decrease, the values during diltiazem action are plotted in the inset of Fig. 3 as

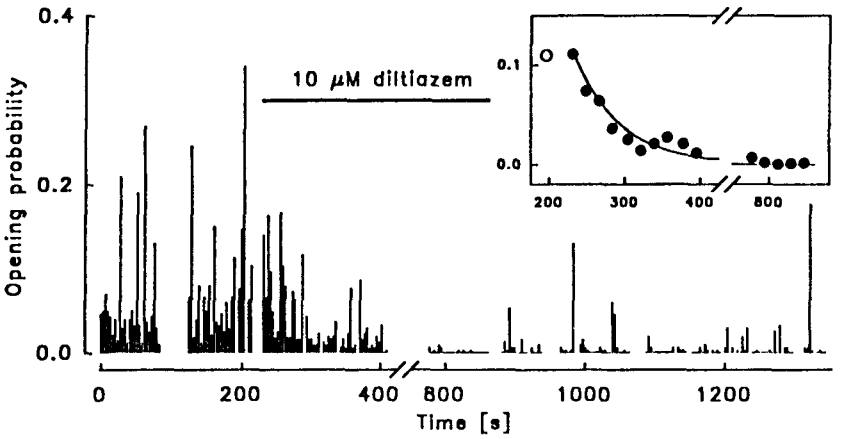

Figure 3. The influence of diltiazem on the probability of the channel being open during the pulse. The open state probability is plotted for every sweep against time from the beginning of the experiment. Diltiazem (10 μmol/l) was present in the bath solution during the period indicated by the horizontal bar. Blank parts correspond to gaps in the measurements; nulls are shown as dots. The open state probability was calculated by adding up the open durations of individual openings in a pulse and dividing the total open time by the number of simultaneously active channels in the patch and by pulse duration. Inset: Determination of the time course of diltiazem action. Every point was calculated as a moving average of 10 sweeps from the data shown in the main figure. The empty and filled circles represent averages immediately before and during perfusion with diltiazem, respectively. The solid line is a monoexponential fit to the data with a time constant of 60.1 s.

moving averages of 10 consecutive sweeps. The effect of diltiazem appears practically immediately after the start of perfusion and the open probability decreases with a time constant $\tau = 60.1$ s. Before the addition of diltiazem the mean open probability in the sweep was 0.079 ± 0.023. In the steady state $\bar{p}$ had fallen down to 0.0016 ± 0.0006. The fall in $\bar{p}$ after application of diltiazem was always highly significant ($\alpha < 0.005$ for testing changes within experiments by Student's t-test).

Diltiazem removal was always accompanied by reappearance of multiple openings and a significant decrease in the number of nulls. Channel open probability in the sweep for the first 10 min of recovery rose five times ($\bar{p} = 0.0083 \pm 0.0022$). First signs of recovery in channel activity came as fast as the onset of diltiazem action, actually not much slower than complete bath perfusion. However, the recovery was never complete. It is not possible to decide whether channel rundown took place or whether diltiazem accumulated in the pipette or intracellular space.

Discussion

We have shown in our experiments that the effect of diltiazem on the open state probability of the L-type calcium channels arises mainly from a decreased number of openings per sweep and from an increased number of sweeps without channel openings. This dominant effect of diltiazem can be interpreted as a result of its interaction with the inactivated state of the channel, although some contribution from interaction with the closed states cannot be ruled out by our multichannel experiments. The inactivated state has already been proposed as the main target for diltiazem by Lee and Tsien (1983) and Uehara and Hume (1985) on the basis of whole-cell experiments. In this respect diltiazem acts similarly as do both dihydropyridines and diphenylalkylamines (Hess et al. 1984; Kokubun et al. 1986; Kawashima and Ochi 1988; and Pelzer et al. 1984).

The ability of diltiazem to interact with the open state of the channel was tested directly by measuring its effects on the mean open time. On the basis of our results we cannot confirm interaction with open Ca channels, as diltiazem does not impose measurable changes in Ca channel mean open time. In this regard it seems to differ from the diphenylalkylamine D-600, which was shown to decrease the mean open time of Ca channels markedly (Pelzer et al. 1984), and to bear more similarities with the dihydropyridine-type antagonists (Hess et al. 1984; Kawashima and Ochi 1988).

Diltiazem did not manifest any effect on the single channel current which was 1.1 pS in 110 mmol/l Ba^{2+} at +10 mV, well in the range reported for the L-type Ca channels (Nilius et al. 1985). The same finding was reported for diphenylalkylamines (Pelzer et al. 1984) and dihydropyridines (Hess et al. 1984; Kawashima and Ochi 1988), and seems to be a common feature for all organic Ca antagonists.

Experiments with multichannel patches allowed us to estimate the rate of access of Ca channels by diltiazem through the membrane phase. When added to the rest of the cell around the isolated patch, its effect developed with an apparently exponential time course. The estimated time constant of 60 s may reflect the diffusion of diltiazem to its binding site. This speed, together with no change of the single channel current amplitude suggest that the interaction develops via the membrane phase rather than through the extracellular lumen of the open channel. Otherwise one should suppose extremely fast diffusion of diltiazem to the pipette lumen across the cell and the patched membrane. We cannot exclude, however, the inhibitory effect of diltiazem to occur via the inner mouth of the channel.

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